Antibody- and Complement-Dependent Cell Injury Assayed by ^{51}Cr Release from Human Peripheral Blood Mononuclear Cells Pretreated with Lipopolysaccharide

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Exposure of human peripheral blood mononuclear (MN) cells to deesterified (alkali-treated) lipopolysaccharide (LPS-OH) and then to ^{51}Cr rendered the cells susceptible to ^{51}Cr release in the presence of specific antibody and complement. The assay was optimized by using rough (Rb$_2$ or Re) LPS. ^{51}Cr release did not occur from cells preexposed to untreated or electrodialyzed LPS. Studies of isolated monocytes and lymphocytes revealed that the majority of the ^{51}Cr released was derived from monocytes. The optimum concentration of LPS-OH was 10 µg/ml. Antiyersinia agglutinin-positive serum, but not a negative serum, obtained from patients with reactive yersinia arthritis caused ^{51}Cr release from MN cells pretreated with yersinia LPS-OH. This implies that during yersinia infection antibodies are generated that can attack the cell membrane–LPS-OH complex. We conclude that the method provides a tool to demonstrate binding of LPS to MN cells in a manner that leads to cell injury in an immune host.

Endotoxin (lipopolysaccharide [LPS]) is a powerful biological effector that participates in the pathogenesis of gram-negative infections by stimulating and modifying a number of host responses (10, 44). On the cellular level, LPS gives rise to proliferation of B cells (4) and cultured fibroblasts (50), production of prostaglandins (26, 45, 51) and interleukin-1 from monocytes and macrophages (15), and tumor cell killing by macrophages (2, 38) and T lymphocytes (47). These responses are believed to be a consequence of an initial binding of LPS to the cell membrane. Such binding has been measured by using isotopically or fluorescence-labeled LPS (2, 6, 18, 52) or fluorescence-labeled antibodies to it (22). It is, however, uncertain to what extent LPS binding measured in this way is functionally relevant. Thus, whereas more LPS was shown to bind to splenic B than T cells of the mouse, paralleling the greater proliferative response of B cells (22, 56), B cells and macrophages of C3H/HeJ mice that do not respond to LPS bound as much LPS and at the same rate as did cells from LPS-responsive mice (6, 34, 44).

Another consequence of LPS binding to animal (or human) cells during infection would be introduction of new antigenic epitopes on the cell surface. This can be demonstrated in vitro by agglutination with LPS-specific antisera or complement- and antibody-dependent lysis of erythrocytes. Such modification of the antigenic properties of various cells during infection could sensitize these cells to antibody formed during the infection and lead to host cell injury even after the infection is cured. The slow catabolism of LPS (9) allows it to persist in the body for weeks coinciding with peak antibody responses. We have now developed a method to assay for such events during postinfectious conditions, e.g., reactive arthritis after gram-negative infections. By this method we can detect antibody and complement-dependent cell injury by ^{51}Cr release from mononuclear (MN) cells sensitized with LPS. In view of the great heterogeneity in molecular size and composition of all LPS preparations

isolated from smooth (S) wild-type bacteria (20, 42), we developed the assay with LPS from rough (R) mutants in which the heterogeneity is much less. We then prepared LPS from smooth *Yersinia enterocolitica* O:3 and could show that sera from patients with reactive arthritis after yersinia enteritis caused release of ^{51}Cr from MN cells preexposed to this LPS.

MATERIALS AND METHODS

Cells. Blood was obtained by venipuncture from healthy volunteers of the laboratory staff, aged 20 to 40 years. Buffy coat cells were separated from heparinized (10 IU/ml, preservative-free heparin; Medica Co., Helsinki, Finland) peripheral blood by dextran (1 ml of 6% dextran per 10 ml of blood; Medipolar, Helsinki, Finland) sedimentation (60 min at 37°C). The MN cell fraction was obtained by Ficoll-Isopaque density gradient centrifugation (5), and monocytes and lymphocytes were separated by discontinuous Percoll density gradient centrifugation (3). The cells were washed two times with RPMI 1640 medium (Orion Diagnostica, Helsinki, Finland) containing 2.5 mg of gelatin (type II; Sigma Chemical Co., St. Louis, Mo.) per ml; the gelatin-RPMI 1640 medium was sterilized by passing it through a 0.22-µm-pore-size membrane filter. Differential cell counts were done on the basis of cell morphology on cytocentrifuge slides after staining by the May-Grunwald-Giemsa technique.

LPS preparation. The bacterial strains used for isolating LPS were IH5300, a fresh clinical isolate of *Y. enterocolitica* O:3 (55), and R mutant strains of chemotypes Re or Rb$_2$ (Fig. 1) (32). Re-LPS was derived from *Proteus mirabilis* R45 (25), *Escherichia coli* D21f2 (33), or *Salmonella minnesota* R595 (31); Rb$_2$ LPS was from *Salmonella typhimurium* SH5014 (40). The yersinia LPS was isolated by the hot phenol-water method (54) from bacteria grown at 22°C, whereas the R-form LPSs were isolated by the phenol-chloroform-petroleum ether method (13) from bacteria grown at 37°C. In
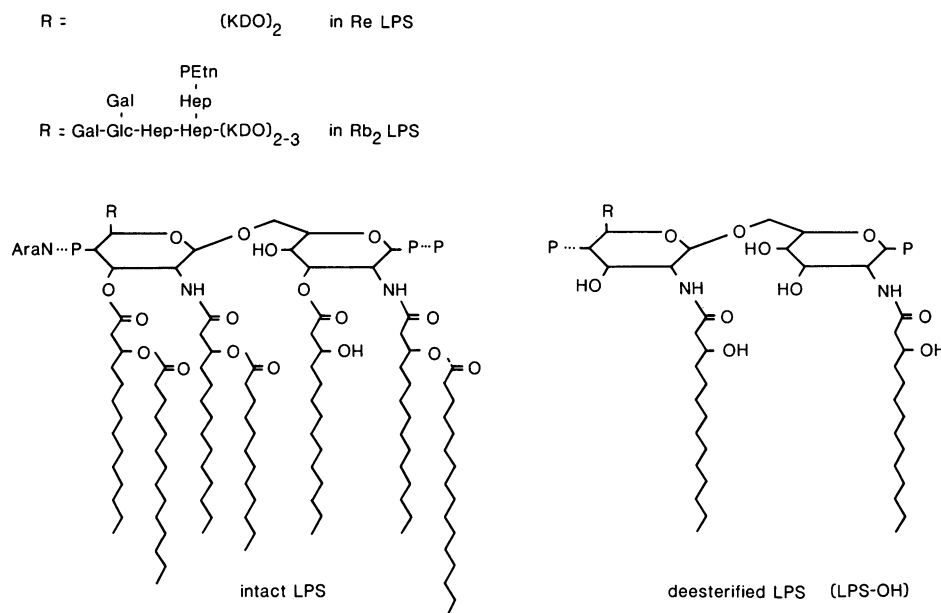
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FIG. 1. Schematic presentation of the structure of Re- and Rb_2-LPSs from salmonellae and their deesterified derivatives. Abbreviations: AraN, 4-aminoarabinose; P, phosphate; Etn, ethanolamine; KDO, 3-deoxy-D-manno-2-octulosonic acid; Hep, heptose; Glc, glucose; Gal, galactose. Fatty acids were 3-hydroxytetradecanoic acid and nonhydroxylated long-chain fatty acids.

some experiments also electrodialyzed LPS was used in the form of its triethylamine salt (11). Deesterified (alkali-treated) LPS (LPS-OH) was prepared by incubating LPS (5 mg/ml) for 1 h at 56°C in 0.2 N NaOH (24); after centrifugation (3,000 ×g) and neutralization by acetic acid, the supernatant was dialyzed overnight.

Sera. All sera were inactivated by heating to 56°C for 30 min and stored at −20°C until use.

Polyclonal immune sera were raised in rabbits. KH 440 was obtained after immunization with a *Chlamydia trachomatis* extract (39) and shown to react specifically in enzyme immunoassay and immunoblots with all Re-LPSs, irrespective of their source (41). KH 172 raised by immunization with a complex of the Rb_2-LPS with porins of *S. typhimurium* reacted with Rb_2 but not with Re-LPS in enzyme immunoassay or immunoblots. KH 556 was obtained after immunization with whole heat-killed bacteria of *Y. enterocolitica* O:3 grown at 22°C; it agglutinated the same bacteria and bound in immunoblots to both the O-antigenic S-type LPS and the R core (55; M. Zaleska, unpublished data).

Convalescent sera were obtained from three patients with reactive arthritis triggered by *Y. enterocolitica* O:3 enteritis, diagnosed on the basis of a typical clinical picture and at least a fourfold change in antiyersinia agglutinin titers (27, 28). Antiyersinia agglutinin titers of these sera were determined as described previously (1). In addition, a pool of normal human sera (NHS) was used.

Exposure of cells to LPS and [51Cr]chromate. *Scheme 1.* Cells (3.0 × 10^6) were suspended in 400 μl of the gelatin-RPMI 1640 medium that had been mixed at a ratio of 10 to 1 (vol/vol) with phosphate-buffered saline (PBS) or LPS in PBS (1 mg/ml to 10 μg/ml). The cells were incubated for 2 h at 22°C, washed two times with gelatin-RPMI 1640 medium, and finally suspended in 400 μl of this medium. [51Cr]chromate (50 μl; 1 mCi/ml; Radiochemical Centre, Amersham, England) was added, and the cell suspension was incubated for 60 min at 37°C on a shaker. After this the cells were

washed two times with gelatin-RPMI 1640 medium and finally suspended in 1.2 ml of this medium.

Scheme 2. To reduce incubation periods and cell washings, LPS and [51Cr]chromate were added at the same time, and the suspension was incubated with shaking for 90 min at 37°C. When cells prepared by the two methods were tested in parallel in the 51Cr release assay (see below), they were similar in both the degree and specificity of 51Cr release (data not shown). Scheme 2 was therefore adopted for use in the latter part of the studies, including the experiments with isolated monocytes, lymphocytes, and sera from patients.

Other modifications were tested. A series of preliminary experiments showed that the addition of 10% (wt/vol) heat-inactivated fetal calf serum or pooled NHS to the RPMI 1640 medium totally abolished the specific 51Cr release from MN cells (data not shown). In RPMI 1640 medium alone the [51Cr]chromate labeling of MN cells was markedly less than in gelatin-RPMI 1640 medium (data not shown). We therefore used the gelatin-RPMI 1640 medium without serum.

51Cr release assay. Samples (50 μl) of the LPS- and [51Cr]chromate-treated cell suspension (1.25 × 10^5 cells) were added to wells of disposable tissue culture trays (MicroTest III; Becton Dickinson Labware, Oxnard, Calif.). Samples (50 μl) of antiserum diluted 1:100 in PBS or PBS only were then added to the wells. After incubation for 30 min at 22°C, 100-μl samples of rabbit serum (pretreated for its complement activity in this assay and stored in small aliquots at −70°C) diluted 1:5 in PBS were added to the wells, and control wells received the same amount of 5% Triton X-100 in distilled water or PBS.The trays were incubated for 60 min at 37°C, after which time the cells were spun down, and 100-μl samples of the cell-free supernatants were aspirated and measured for radioactivity (counts per minute). The tests were performed in triplicate, and the arithmetic mean ± the standard deviation of the counts per minute was calculated. 51Cr release percentage was determined by the formula 100 × $[(a − b)/(c − b)]$, where a is the mean counts per minute released in the presence of both

TABLE 1. Binding of LPS (10 μg/ml) to MN cells demonstrated by ^{51}Cr release after treatment with specific antibody and complement (C)

Treatment[a]	[51]Cr (cpm, mean ± SD) released by:						
	Triton X-100	PBS	C	Anti-Re		Anti-Rb₂	
				+C	−C	+C	−C
No LPS	2,986 ± 44	307 ± 87	308 ± 84	315 ± 28	336 ± 49	285 ± 40	305 ± 50
Re-LPS	2,195 ± 167	308 ± 37	411 ± 77	484 ± 27	354 ± 33	293 ± 9	339 ± 21
Re-LPS-OH	2,335 ± 99	264 ± 5	334 ± 29	878 ± 46	401 ± 90	323 ± 28	343 ± 7
Rb₂-LPS	2,694 ± 153	297 ± 62	366 ± 47	346 ± 17	328 ± 22	518 ± 46	359 ± 13
Rb₂-LPS-OH	2,352 ± 25	392 ± 28	463 ± 1	488 ± 39	447 ± 49	1,084 ± 13	586 ± 19

[a] MN cell treatment was done by scheme 1 (see Materials and Methods); Re-LPS was from *S. minnesota*, and Rb₂-LPS was from *S. typhimurium*; the corresponding deesterified LPS-OH forms were prepared by treatment with 0.2 N NaOH.

antiserum and complement, in the presence of complement only, or in the presence of antiserum only; *b* is the mean counts per minute released spontaneously in PBS; and *c* is the mean counts per minute released after lysing the cells with Triton X-100.

RESULTS

[51]Cr **release from MN cells pretreated with LPS.** The basic assay was worked out with MN cells, using Re- and Rb₂ LPSs from *Salmonella* species and antisera specific to these. MN cells were exposed to LPS derivatives at concentrations of 100, 10 (Table 1), or 1 μg/ml and thereafter to [51]Cr. Neither serum caused ^{51}Cr release from cells not exposed to LPS or LPS-OH. Pretreatment of the cells with either Re- or Rb₂-LPS, followed by antiserum and complement, caused a minimal ^{51}Cr release only (Table 1). However, pretreatment with deesterified LPS (LPS-OH) resulted in a marked, antiserum-specific ^{51}Cr release in the presence of complement. Complement alone, without added antibody, had little effect. Pretreatment with either 10 or 100 μg/ml of LPS-OH provided a good ^{51}Cr release, whereas 1 μg/ml was suboptimal (data not shown; see also Table 3). The addition of 10% serum to the medium during LPS or LPS-OH treatment (Re or Rb₂) abolished all ^{51}Cr release (data not shown).

To test for the requirements of LPS-OH binding, we studied the ^{51}Cr release from MN cells preexposed to Re-LPSs derived from *P. mirabilis*, *E. coli*, and *S. minnesota* which have small differences in the phosphate or fatty acid substitutions or both (46). The triethylamine salt of electrodialyzed LPS was included in these assays to test for the importance of LPS solubility. None of the intact LPSs, electrodialyzed or not, induced ^{51}Cr release from the MN cells, but all the deesterified LPS (LPS-OH) provided good release in the presence of specific antiserum (Table 2). The addition of complement enhanced the ^{51}Cr release in each case.

Which cells are responsible for ^{51}Cr release? The MN cells were fractionated into monocytes and lymphocytes and assayed for ^{51}Cr release after pretreatment with Re-LPS-OH (Table 3). The controls (detergent-lysed cells) indicated that monocytes bound so much more ^{51}Cr than the lymphocytes that the ^{51}Cr release observed with the MN fraction was almost exclusively due to release from monocytes.

In the presence of both antiserum and complement, specific ^{51}Cr release occurred from monocytes preexposed to 100 or 10 μg of Re-LPS-OH per ml but not from those exposed to 1 μg/ml (Table 3). Antiserum without complement also induced ^{51}Cr release from monocytes treated with 100 or 10 μg of LPS per ml, although the release percentage was less than that in the presence of both antiserum and

complement. The ^{51}Cr release from monocytes was antibody specific both in the presence of complement and in the absence of it. Complement alone did not release [51]Cr.

Lymphocytes preexposed to 100 μg of the Re-LPS-OH per ml released ^{51}Cr activity in the presence of complement even without specific antiserum (Table 3), possibly indicating injury caused by this concentration of LPS-OH (note the low amount of ^{51}Cr recovered from these cells by Triton X-100). Lymphocytes preexposed to 10 μg of Re-LPS-OH per ml showed a specific, complement-dependent ^{51}Cr release, whereas those exposed to 1 μg/ml showed no ^{51}Cr release (Table 3). Antiserum to Re-LPS did not induce ^{51}Cr release from lymphocytes in the absence of complement.

[51]Cr **release by sera of patients with yersinia arthritis.** We then asked whether antibodies detectable by the ^{51}Cr release assay are generated in vivo in response to infection and present during a postinfectious reactive arthritis. MN cells pretreated with LPS-OH from *Y. enterocolitica* O:3 in the same manner as used above for Rb₂- or Re-LPS-OH released ^{51}Cr after treatment with antiyersinia, but not anti-Re or anti-Rb₂ (Table 4). Postinfectious sera were obtained from patients with reactive arthritis after yersinia enteritis. Three sera that contained antiyersinia agglutinins indeed caused ^{51}Cr release from MN pretreated with yersinia LPS-OH,

TABLE 2. Binding of different Re-LPS derivatives (10 μg/ml) to MN cells assayed by ^{51}Cr release assay

Treatment[a]	% [51]Cr release[b]			
	Anti-Re		Anti-Rb₂	
	+C	−C	+C	−C
No LPS	2	2	2	4
P. mirabilis				
Re-LPS	0	2	0	3
Re-LPS-ED[c]	0	0	0	0
Re-LPS-OH[c]	29	15	2	3
E. coli				
Re-LPS	1	0	1	5
Re-LPS-ED	0	0	0	0
Re-LPS-OH	42	14	2	2
S. minnesota				
Re-LPS	5	3	2	3
Re-LPS-ED	3	4	2	3
Re-LPS-OH	21	13	3	5

[a] MN cell treatment was done by scheme 1 (see Materials and Methods).
[b] Less than 7% in the presence of complement (C) or antiserum only.
[c] LPS-ED is electrolyzed LPS as the triethylamine salt; LPS-OH is LPS deesterified by treatment with 0.2 N NaOH.

TABLE 3. [51]Cr release from the MN cell fraction and separated monocytes and lymphocytes exposed to Re-LPS-OH[a] and then to antiserum or complement (C) or both

Treatment[b] of cells (1.25 × 10^{5}/assay) with LPS (μg/ml)	[51]Cr (cpm, mean ± SD) released by Triton X-100	% [51]Cr release				
		Anti-Re		C	Anti-Rb₂	
		+C	−C		+C	−C
MN cell fraction						
No LPS	2,619 ± 9	1	2	2	4	4
LPS-OH (10)	3,478 ± 232	41	17	2	2	3
Monocytes						
No LPS	13,284 ± 340	1	1	0	0	2
LPS-OH (100)	13,882 ± 595	24	18	0	0	0
LPS-OH (10)	11,229 ± 175	18	13	1	0	3
LPS-OH (1)	13,304 ± 509	3	1	0	0	2
Lymphocytes						
No LPS	1,680 ± 135	0	0	0	0	0
LPS-OH (100)	386 ± 14	64	6	22	20	6
LPS-OH (10)	1,383 ± 127	26	4	1	1	1
LPS-OH (1)	1,106 ± 58	1	2	0	2	0

[a] The LPS was isolated from *S. minnesota*.

[b] Cell treatment was done by scheme 2 (see Materials and Methods); the MN cell fraction was 66% lymphocytes and 26% monocytes, separated monocytes were 92% monocytes and 5% lymphocytes, and separated lymphocytes were 98% lymphocytes and 1% monocytes.

whereas no such release was observed with pooled NHS (Table 5).

DISCUSSION

The results in the present study show that ^{51}Cr release occurs from labeled human peripheral blood MN cells first exposed to an appropriate LPS preparation and then to LPS-specific antiserum and complement. The ^{51}Cr release was specific to the LPS-OH applied and can thus be taken as a measure of LPS-OH binding in a way that could lead to functional consequences even in vivo during infection. The results also show that antiyersinia agglutinin-positive serum, but not a negative serum, obtained from patients with reactive arthritis after yersinia enteritis induced ^{51}Cr release from MN cells pretreated with yersinia LPS-OH. This implies that during yersinia infection antibodies are generated that can attack the cell membrane–LPS-OH complex and may thereby mediate a host cell injury.

The binding of ^{51}Cr was dependent on the target cell type. Monocytes bound 10 times more ^{51}Cr than did lymphocytes and were the main MN cells responsible for the LPS-dependent ^{51}Cr release. [51]Cr release from the monocytes was

TABLE 4. [51]Cr release induced by LPS-OH derived from *Y. enterocolitica* O:3

Pretreatment of MN cells (μg/ml)	% [51]Cr release[a]					
	Antiyersinia		Anti-Re		Anti-Rb₂	
	+C	−C	+C	−C	+C	−C
Y. enterocolitica						
LPS-OH (100)	52	8	5	1	5	0
LPS-OH (10)	45	17	4	1	4	1
LPS-OH (1)	11	1	3	1	4	1
P. mirabilis						
Re-LPS-OH (100)	2	0	33	4	4	0
Re-LPS-OH (10)	3	1	42	24	4	1
Re-LPS-OH (1)	3	0	14	8	4	1
No LPS	4	1	4	1	4	1

[a] [51]Cr release in the presence of complement (C) only was 2 to 4% (range).

observed even in the absence of added complement but required the presence of the specific anti-LPS; it could be due to complement components synthesized in situ by the monocytes (36).

Intact Re-LPS, known to occur as large micelles (11), did not bind significantly on MN cells as measured by ^{51}Cr release, whereas deesterified LPS (LPS-OH) did. The deesterification (alkali treatment) reduces the number of fatty acids of LPS from approximately seven to two per molecule and thereby increases the hydrophilicity of the LPS (Fig. 1). Alkali treatment also reduces the number of charged groups in LPS and thereby decreases ionic interactions between LPS molecules (12); both effects are likely to reduce micelle formation. The LPS-OH in fact behaves like a detergent, and thus has a tendency to bind in membranes and liposomes (23). Electrodialysis of LPS also decreases LPS aggregation (11) and allows it to bind to erythrocytes (14); however, it did not allow the Re-LPSs to bind to MN cells as determined by the ^{51}Cr release assay.

In many studies the binding of LPS has been assayed by fluorescence or radiolabeling methods (2, 6, 18, 52). It is quite possible that these experiments detect loose binding of LPS micelles without integration into the membrane. For example, studies with *E. coli* O:111 have suggested that

TABLE 5. [51]Cr release induced by sera from patients with yersinia arthritis and by NHS

Source of serum	Antiyersinia agglutinin titer	% [51]Cr release[a] with a serum dilution of:			
		1:4		1:16	
		+C	−C	+C	−C
Patient 1	1:1,280	17	12	7	1
Patient 2	1:1,280	22	15	16	12
Patient 3	1:1,280	22	15	16	8
Pooled NHS	<1:40	1	0	1	0

[a] [51]Cr release was 2% in the presence of complement (C) only. It was 51% in the presence of both complement and rabbit antiyersinia serum and 5% in the presence of rabbit antiyersinia serum only. MN cells from a healthy volunteer had been preexposed to yersinia LPS-OH (100 μg/ml).

R-form LPS binds more avidly than S form (16), rather in contrast to what is expected on the basis of solubility but consistent with intact micelles remaining in superficial contact with the cell membrane.

The alkali treatment that removes the ester-linked fatty acids from LPS also abolishes or greatly reduces many of its endotoxic effects, including mitogenicity, lethal toxicity, pyrogenicity, production of prostaglandins, and development of cytotoxicity (30, 38, 43). The deesterified LPS is, however, not without biologial effects. It has been shown to inhibit natural killer activial from human peripheral blood MN cells (48), and as shown in this paper, it can very effectively modify the cell and make it a target for antibody-mediated lysis (14), which would have a variety of consequences during the postinfection period. This kind of LPS modification and subsequent antibody binding could also render the cells susceptible to antibody-dependent cell-mediated cytotoxic injury.

Could binding of LPS as shown here with LPS-OH take place in vivo during or after infection? All gram-negative bacteria have LPS as a major component of their outer membrane, and they release LPS, often in large quantities, during growth or on exposure to Tris-EDTA or polycationic agents (7, 21, 29, 37, 41, 49, 53). In the host, LPS is slowly degraded, e.g., by liver cells (9), polymorphonuclear leukocytes (19), and macrophages (35). The degraded LPS has a reduced amount of fatty acids and is therefore more likely to integrate in cell membranes. LPS extracted from macrophages after uptake of intact E. coli has been shown to be a potent immunostimulant (8). The ability of such biodegraded LPS to interact with mammalian cells has not to our knowledge been determined. Even if released in free form, the LPS is likely to bind to serum proteins, and this binding is known to reduce greatly its interaction with cells. On the other hand, serum components may not always be present at the foci of infection, where the bacteria are in direct contact with host cells; for example, the effects of LPS on ciliary action of neighboring cells in a local gonococcal infection have been demonstrated (17).

In summary, we demonstrated a possible mechanism by which LPS could lead to immunologically mediated postinfectious sequelae in humans. We also characterized the basic parameters of this LPS-cell interaction. The assay system described should be useful in a further characterization of these interactions in the more complex situation occurring during infection. The added complexities that should be taken into account would be the heterogeneity of the LPS produced by virulent bacteria, the form of LPS in the outer membrane or when released from it, the processing of the LPS by tissue cells, and the presence of both antibody and immune T lymphocytes. The postinfectious reactive arthritis after infection with, e.g., *Y. enterocolitica* or *C. trachomatis* is a condition in which all these mechanisms might be operative.

ACKNOWLEDGMENTS

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